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Layered Structure of Bacterial Aggregates Produced in an Upflow Anaerobic Sludge Bed and Filter Reactor

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The ultrastructure of bacterial granules that were maintained in an upflow anaerobic sludge bed and filter reactor was examined. The reactor was fed a sucrose medium, and it was operated at 35°C. Scanning and transmission electron microscopy revealed that the granular aggregates were three-layered structures. The exterior layer of the granule contained a very heterogeneous population that included rods, cocci, and filaments of various sizes. The middle layer consisted of a slightly less heterogeneous population than the exterior layer. A more ordered arrangement, made up predominantly of bacterial rods, was evident in this second layer. The third layer formed the internal core of the granules. It consisted of large numbers of *Methanothrix*-like cells. Large cavities, indicative of vigorous gas production, were evident in the third layer. On the basis of these ultrastructural results, a model that presents a possible explanation of granule development is offered.

Anaerobic digestors are commonly used in the treatment of municipal and industrial wastewaters. Fairly recently, anaerobic upflow systems have been applied to the treatment of wastewaters. The upflow anaerobic sludge blanket reactor was developed by Lettinga and co-workers (20). A variant of this design was subsequently produced by Guiot and van den Berg (14) and is referred to as an upflow anaerobic sludge bed and filter system. A major advantage of these upflow systems is that their design permits the retention of a greater amount of active biomass in comparison with other anaerobic reactors. It is known that the loading rate of an anaerobic wastewater treatment system is dependent on the amount of active biomass present in the reactor (19). Therefore, these upflow systems can accommodate organic loading rates several times higher than those of other anaerobic digestors. The ability of the upflow reactors to accumulate large amounts of biomass is due to the adhesion of bacterial cells to each other. The adhering bacteria form granules of biomass which can be several millimeters in diameter.

The adhesion of bacteria to inert surfaces and the subsequent biofilm development have received considerable attention (8, 18, 24). In aquatic environments, adhesion to a surface is thought to provide the colonizing organisms with a supply of nutrients, since inorganic and organic nutrients are concentrated at this interface (15). Furthermore, once they adhere to a surface which is contacted by flowing water, the bacteria are exposed to a high concentration of nutrients because of a filtration effect (12). Adhesion also functions to maintain microorganisms in a nutritionally favorable environment in upflow anaerobic sludge blanket and upflow anaerobic sludge bed and filter reactors in which there are no inert substrata available (excluding the walls of the reactor). In this situation, the upflow velocity will select for organisms which can adhere to each other to form well-settling granular sludge. The granular sludge will remain in the reactor and will then be exposed to the continuous supply of nutrients which is injected into the bottom of the reactor.

The metabolic reactions which occur during anaerobic digestion also suggest why the aggregation of microorganisms into granules would be advantageous. The degradation of complex substrates into methane and carbon dioxide during anaerobic digestion involves the interaction of at least

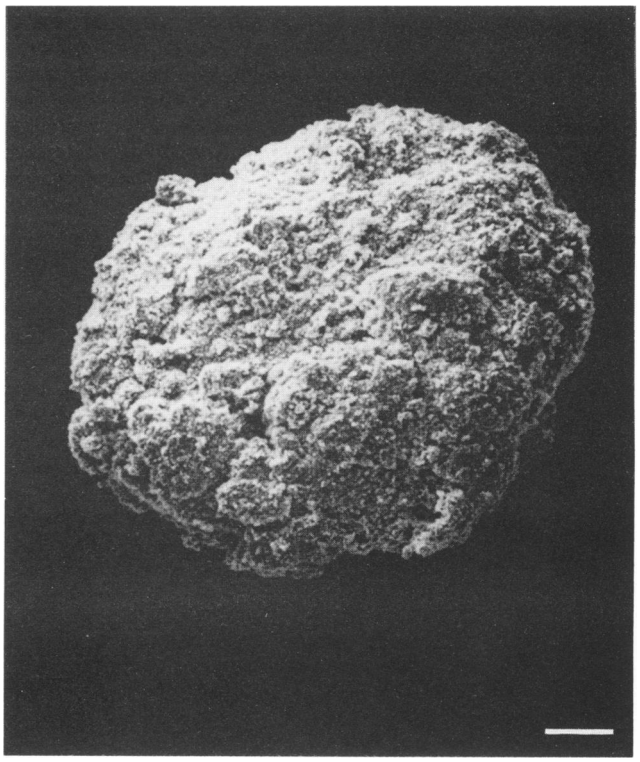

FIG. 1. Scanning electron micrograph showing the surface topography of an entire granule. Bar, 100 μm.

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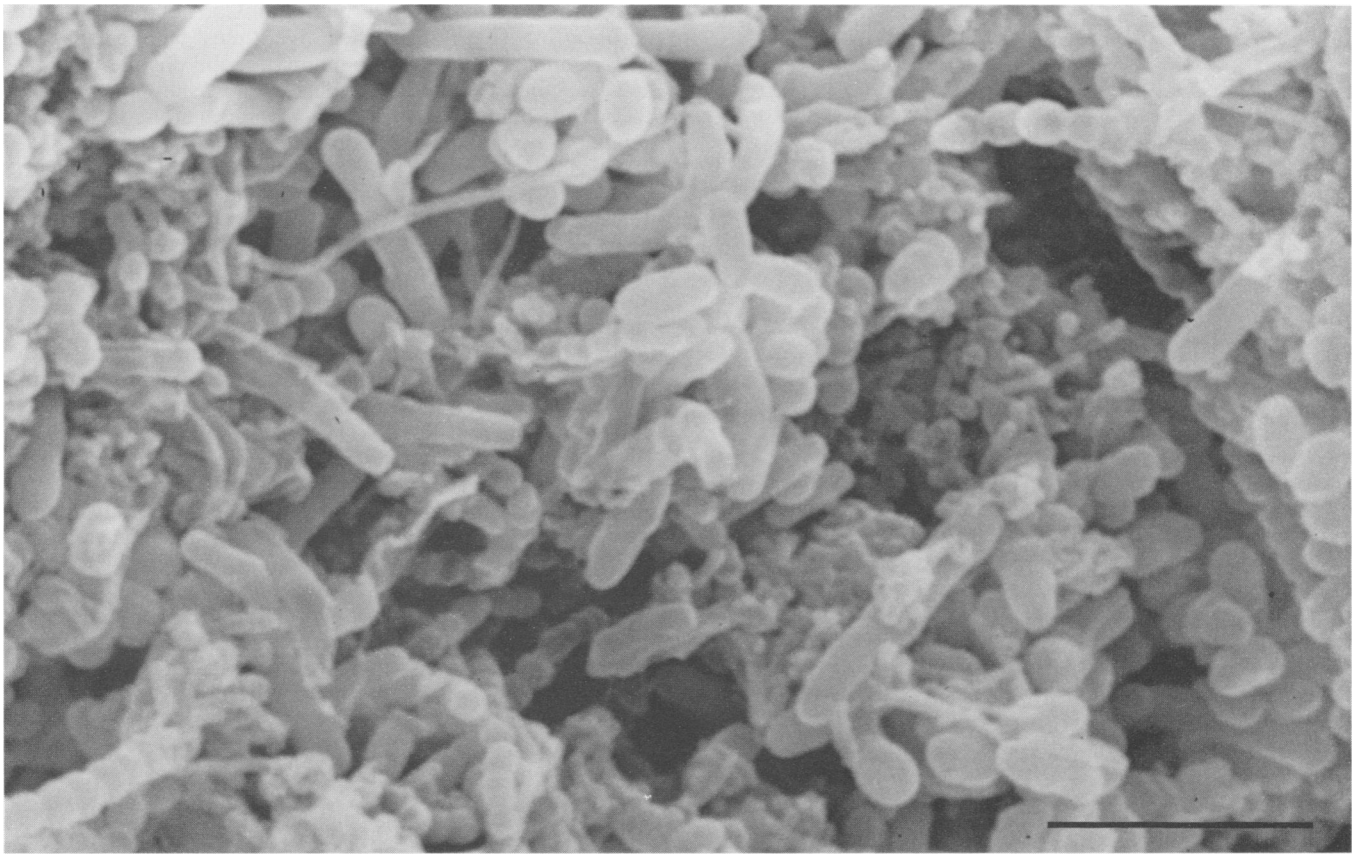

FIG. 2. Scanning electron micrograph showing that the granule surface is colonized by a mixed population that includes long rods, chain-forming cocci, thin filaments, and small rods and cocci. Bar, 5.0 μm.

three metabolic groups (17, 22). The first group of fermentative bacteria, the acidogens, conducts the initial degradation of biopolymers. The acids and alcohols so produced are utilized by a second group of organisms called the acetogenic bacteria. The third group of bacteria are the methanogens. Located at the end of the nutrient cascade, methanogens convert CO_2 and H_2, acetate, and a few other simple compounds to methane. Clearly, the close association of members of these three groups in a layered granular structure would ensure a high level of metabolic activity.

The aggregation of bacteria into granular sludge would also be conducive to the creation of cell associations that are obligatory for the utilization of certain substrates. For example, the degradation of propionate (4) and butyrate (11) is thermodynamically unfavorable, unless the hydrogen produced during the oxidation of these two substrates is removed by an H_2-consuming species. Close associations between organisms participating in interspecies hydrogen transfer were observed in the miniaggregates that formed in the liquid phase of a coculture of a butyrate oxidizer and an H_2-consuming methanogen (23). Thus, it was not surprising when the close juxtaposition of a propionate degrader and an H_2-consuming methanogen was shown to occur in granular material (10).

This study was conducted to further elucidate the microbial structure of granules. The information so obtained may indicate which morphotypes play important roles in the induction and development of granular sludge.

MATERIALS AND METHODS

Reactor. Samples of granular sludge were obtained from an upflow sludge bed filter as described by Guiot and van den Berg (14). Granular sludge used to start up the reactor was obtained from an upflow anaerobic sludge blanket reactor that was used to treat cheese whey wastewater (Agropur Coopérative Alimentaire, Notre-Dame du Bon Conseil, Québec, Canada). The reactor had a working volume of 13.5 liters, and it was operated at 35°C. The dilution rate was 2.3 day^{-1}. A 10:1 ratio of recirculation to feed flow yielded a liquid upflow velocity of 0.9 m/h. The specific organic loading rate was 1.3 g of chemical oxygen demand per g of volatile suspended solids per day, with an 82% substrate removal efficiency. The composition of the feed medium (in milligrams per liter) was as follows: $C_{12}H_{22}O_{11}$, 4,400; yeast extract, 44; KH_2PO_4, 88; K_2HPO_4, 118; $(NH_4)_2SO_4$, 221; $KHCO_3$, 5,000; $NaHCO_3$, 4,000 (final pH, 7.2 ± 0.1). This reactor was in operation for 1 month before the samples were collected.

Metabolic activity assays. Granular sludge was collected under anaerobic conditions by using nitrogen gas. To remove any nongranular biomass, the sludge was washed three times with an anaerobic salts solution consisting of (in grams per liter) $Na_2HPO_4 \cdot 7H_2O$, 10.185; K_2HPO_4, 8.01; $NaH_2PO_4 \cdot H_2O$, 2.21; cysteine hydrochloride, 0.125; $Na_2S \cdot 9H_2O$, 0.125; and resazurin, 0.001 (final pH, 7.2 ± 0.1). The granular sludge was suspended to its original volume in the

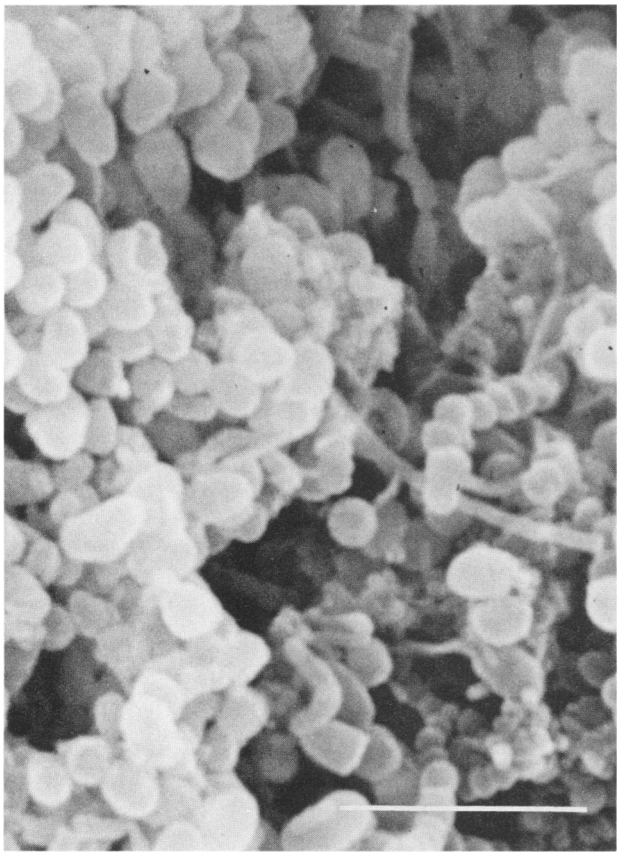

FIG. 3. Surface area consisting of a large microcolony of cocci in association with other structures. Bar, 5.0 μm.

anaerobic salt solution, and 10-ml volumes were transferred to serum bottles which were sealed with oxygen-impermeable butyl rubber stoppers and aluminum seals (Wheaton Scientific, Millville, N.J.). The activity assays were conducted in triplicate at 35°C. Either 5 mg of propionate, 10 mg of formate, 30 mg of acetate, or 30 mg of glucose was added to each serum bottle. The disappearance of acetate and propionate was monitored by gas chromatography, as described by Arcand et al. (2). Formate and glucose were measured by high-pressure liquid chromatography with an SP8100 XR liquid chromatograph (Spectra-Physics, San Jose, Calif.). Formate and glucose were detected with a 3-cm-long Polypore H RP-8 resin (5-μm-diameter) column (Brownlee, Santa Clara, Calif.) combined with a 25-cm Polypore H RP-8 resin (5-μm-diameter) column (Brownlee). The column temperature was 40°C, and the solvent (0.01 N H_2SO_4) had a flow rate of 0.35 ml/min. Formate and glucose were detected by differential refractometry (Spectra-Physics SP 6040 XR).

Transmission electron microscopy. The washed granules were placed in 60-ml serum bottles (Chromatographic Specialties, Inc., Brockville, Ontario, Canada) which contained 5% glutaraldehyde and 0.15% (wt/vol) ruthenium red (Sigma Chemical Co., St. Louis, Mo.) in anaerobic cacodylate buffer consisting of (in grams per liter) $Na(CH_3)_2AsO_2 \cdot 3H_2O$, 16.0; cysteine hydrochloride, 0.125; $Na_2S \cdot 9H_2O$, 0.125; and resazurin, 0.001 (final pH, 7.2 ± 0.1). The serum bottles were sealed with oxygen-impermeable butyl rubber stoppers and aluminium seals (Wheaton Scientific), and fixation was completed overnight at 4°C. The granules were enrobed in 4% agar, washed five times with cacodylate buffer containing 0.05% ruthenium red, and postfixed for 2 h in 2% osmium tetroxide. Samples were washed five times in cacodylate buffer, dehydrated in a water-acetone series, and given two exposures of propylene oxide for 10 min. The samples were then infiltrated with 3:1 propylene oxide-Spurr resin (J. B. EM Services Inc., Dorval, Quebec, Canada) overnight and then given an additional 6 h of exposure to fresh propylene oxide-Spurr resin (3:1). The granules were placed in fresh resin overnight and then embedded in resin for 18 h at 60°C. Thin sections were cut with a glass knife, placed on 200-mesh copper grids, and stained with 1% uranyl acetate (pH 5.0) and lead citrate as described by Reynolds (26). Preparations were examined with a Hitachi 601 transmission electron microscope operating at an accelerating voltage of 50 kV.

Scanning electron microscopy. The washed granules were placed in sealed 50-ml serum bottles which contained 5% glutaraldehyde in anaerobic cacodylate buffer, and fixation was executed overnight at 4°C. To obtain cleaved preparations of the granules, the fixed samples were quick-frozen in liquid nitrogen and cleaved with a mortar and pestle. Whole and cleaved granules were dehydrated through graded series of water-ethanol and ethanol-Freon 113. The samples were placed on aluminum specimen mounts, coated with gold-palladium, and examined with a Hitachi S-450 scanning

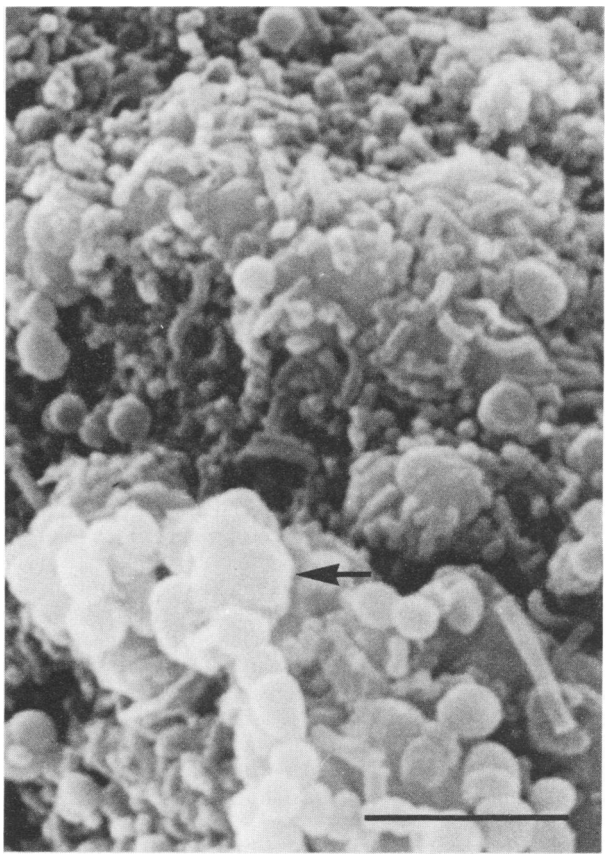

FIG. 4. Small rods and cocci are the predominant organisms in this area of the surface. A collapsed extracellular polymer is evident around the large coccus (←). Bar, 5.0 μm.

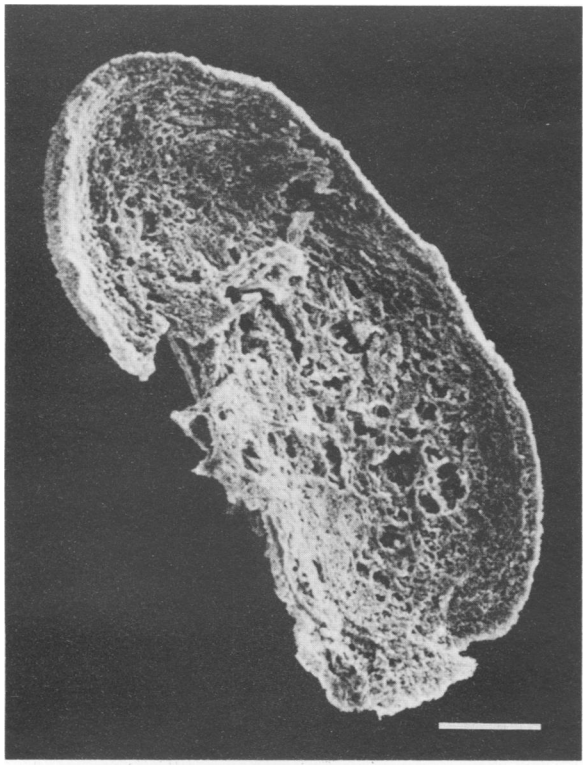

FIG. 5. Cleavage of a granule reveals internal structure and a large number of cavities. Bar, 50.0 μm.

electron microscope operating at an accelerating voltage of 20 kV.

RESULTS

Metabolic activity. Microbial populations capable of utilizing formate, acetate, propionate, and glucose were detected in the granular sludge samples. The specific activities of these substrates (millimoles per gram of volatile suspended solids per day, presented as the means of triplicate samples ± standard deviations) were as follows: glucose, 37.74 ± 10.54; propionate, 1.97 ± 0.56; acetate, 5.00 ± 0.76; and formate, 47.30 ± 5.37. Since the granular sludge had been washed three times prior to testing, organisms present in the reactor medium, as well as those not firmly attached to the granules, were removed and therefore were not responsible for the observed activity. The four substrates tested varied in chemical complexity and thus represented four steps in the degradation of complex substrates into simple products during anaerobic digestion.

Scanning electron microscopy. (i) Granule surface. The granules were irregular spheres (Fig. 1). A close examination of the surfaces of 25 granules revealed the presence of a large diversity of bacterial morphotypes. These included chain-forming coccoid organisms (approximately 0.7 μm in diameter), large rods (1.0 by 2.0 μm), long thin filaments (approximately 0.27 μm in width), and small rods and cocci (less than 1.0 μm in length or diameter) (Fig. 2). Other surface areas (Fig. 3) contained large numbers of clustering coccoid organisms (approximately 1.1 μm in diameter) that resembled members of the order *Methanococcales*. Still other areas (Fig. 4) were covered with large numbers of small rods (0.28 to 0.37 by 0.7 to 0.9 μm) and small cocci (0.23 to 0.47 μm in diameter). A few chain-forming rods (approximately 0.6 by 1.6 μm) which exhibited angular shapes similar to those of *Methanothrix* spp. were occasionally observed on the surface. A collapsed extracellular polymer was observed to be associated with the bacteria on the surface, particularly with the large coccoid organisms (Fig. 4).

(ii) Interior of granules. Quick-freezing and cleavage of 25 granules revealed an internal structure which consisted of a central core of bacterium-encased cavities surrounded by several distinct layers of bacteria (Fig. 5 and 6). The exterior layer was approximately 10 to 20 μm thick (Fig. 7). Not surprisingly, it contained the cell structures observed on the outside surfaces of whole granules. Large *Methanococcales*-like organisms, chain-forming cocci, large long rods, thin filaments, and smaller coccoid organisms and rods were present throughout this layer. Gas spaces often appeared to separate this layer from the underlying one. Thin filamentous organisms, approximately 0.25 μm in width, were one of the predominant bridging species in these spaces. The second layer was a tightly packed structure of bacteria embedded in extracellular polymer (Fig. 7 and 8). Like the exterior layer, the second layer was approximately 10 to 20 μm thick. Various rods appeared to be the predominant structures. The third layer consisted of large microcolonies that were composed almost exclusively of angular-shaped rods (0.4 to 0.5 by 1.0 to 1.8 μm) which possessed a structure similar to those of *Methanothrix* spp. (Fig. 8). These bacteria were

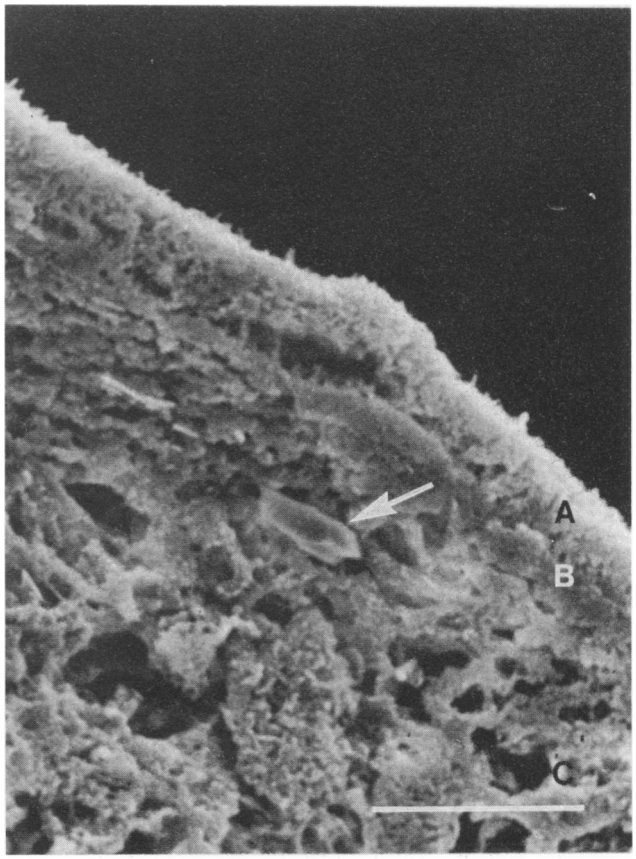

FIG. 6. High magnification of Fig. 5, showing distinct layers (A, B, C) in the granular cross section. The internal cavities are surrounded by bacterial matrices. Inorganic crystals (→) are also noted. Bar, 50.0 μm.

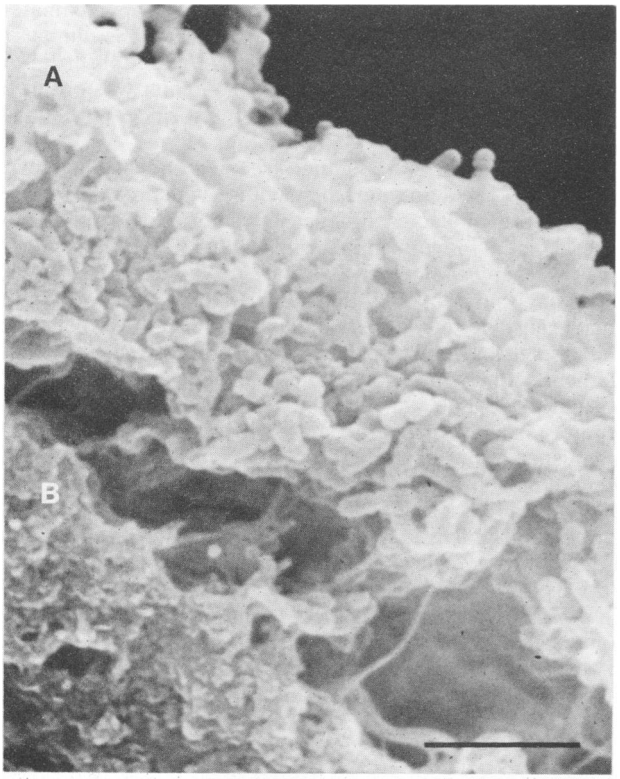

FIG. 7. Detailed examination of the exterior layer (A) reveals a heterogeneous population of rods, filaments, and cocci. The second layer (B) exhibits a tight packing of biomass. Bar, 5.0 μm.

surrounded by an extracellular polymer and formed the walls of cavities that had diameters of 2.5 to 40 μm (Fig. 6 and 9). It is also clearly evident that the cells of this species were arranged in characteristic angular packing (Fig. 10). These bacteria did not always fully separate after cell division, and often they were oriented in stellar and other apparently organized patterns.

Transmission electron microscopy. Species diversity and community structure were also examined by using transmission electron microscopy of thin sections. Sections obtained near the surface contained a diversity of shapes (Fig. 11). Rods and cocci of various sizes, with either gram-positive or gram-negative cell wall structures, were observed. Because of different section planes, it was not always possible to distinguish between the two shapes. Sections obtained from a depth of approximately 20 μm often contained regions in which two or three species were juxtaposed. For example, Fig. 12 shows a gram-positive organism (approximately 0.8 by 1.2 μm) that was closely associated with an electron-dense rod (approximately 0.25 by 0.9 μm). At a depth of 50 to 100 μm, the bacteria predominantly exhibited one shape (Fig. 13). The cells were rod shaped, with a width of 0.4 to 0.5 μm and an average length of 2.5 μm. Short chains of two or three cells were sometimes observed. The presence of different section planes at this depth confirms the scanning electron microscopy observation that these cells were arranged in what could be described as angular packing. The cell envelopes of these bacteria were quite distinct (Fig. 14). The cell membrane was bordered by a thin inner wall. An amorphous layer separated the inner wall from the outer wall. Cells in filaments were separated from each other by septa. This unique cell envelope structure is characteristic of strains of the archaebacterial genus *Methanothrix*.

DISCUSSION

Our findings show that the granules obtained from a mesophilic reactor, which was fed over a 1-month period with a sucrose medium, exhibited a three-layered structure and that each layer possessed a distinguishing morphology. The exterior layer contained a variety of organisms, including clusters of large coccoid organisms (Fig. 3) which strongly resembled methanogenic cocci of the order *Methanococcales* (30). Thin filaments similar to *Methanospirillum* spp. were also present (31). The second layer consisted of a large number of rod-shaped bacteria. Among these rods was a very electron-dense organism (Fig. 12) which resembled a *Methanobrevibacter* sp. and a larger short rod which resembled a *Syntrophobacter* sp. as described by Dubourguier et al. (10). The third layer contained large numbers of rods with flat ends (Fig. 8–10). Scanning electron micrographs revealed that these bacteria occupied large areas of the center of the granules. These bacteria were the same size and shape as previously described *Methanothrix* spp. (9). Transmission electron microscopy provided further discriminating evidence with respect to this morphotype. The presence of two wall layers and the angular shape are very distinctive features (Fig. 14). Similar morphological characteristics were presented previously for *Methanothrix concilii* (25) and

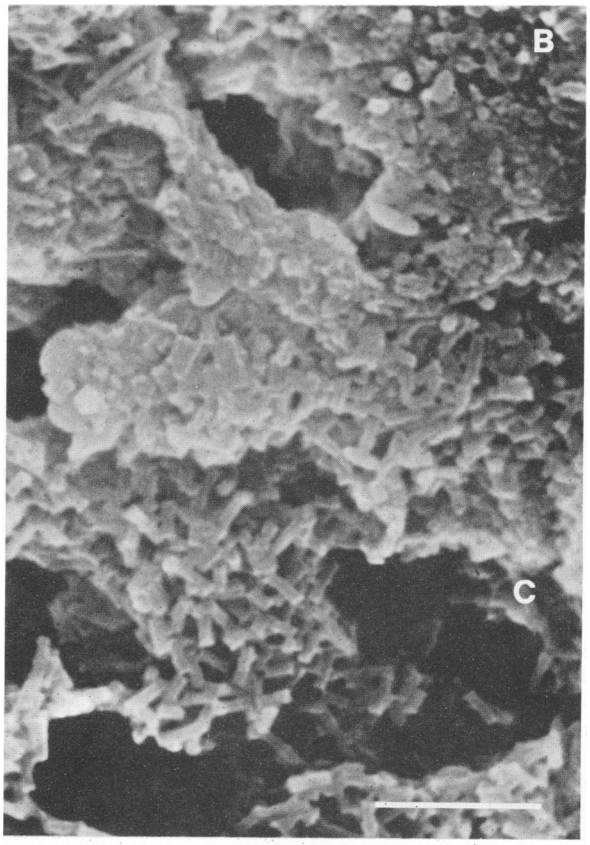

FIG. 8. Second layer (B), consisting predominantly of rods which appear to be embedded in extracellular polymer. The third layer (C) contains principally one morphotype, which forms the walls of the internal cavities. Bar, 5.0 μm.

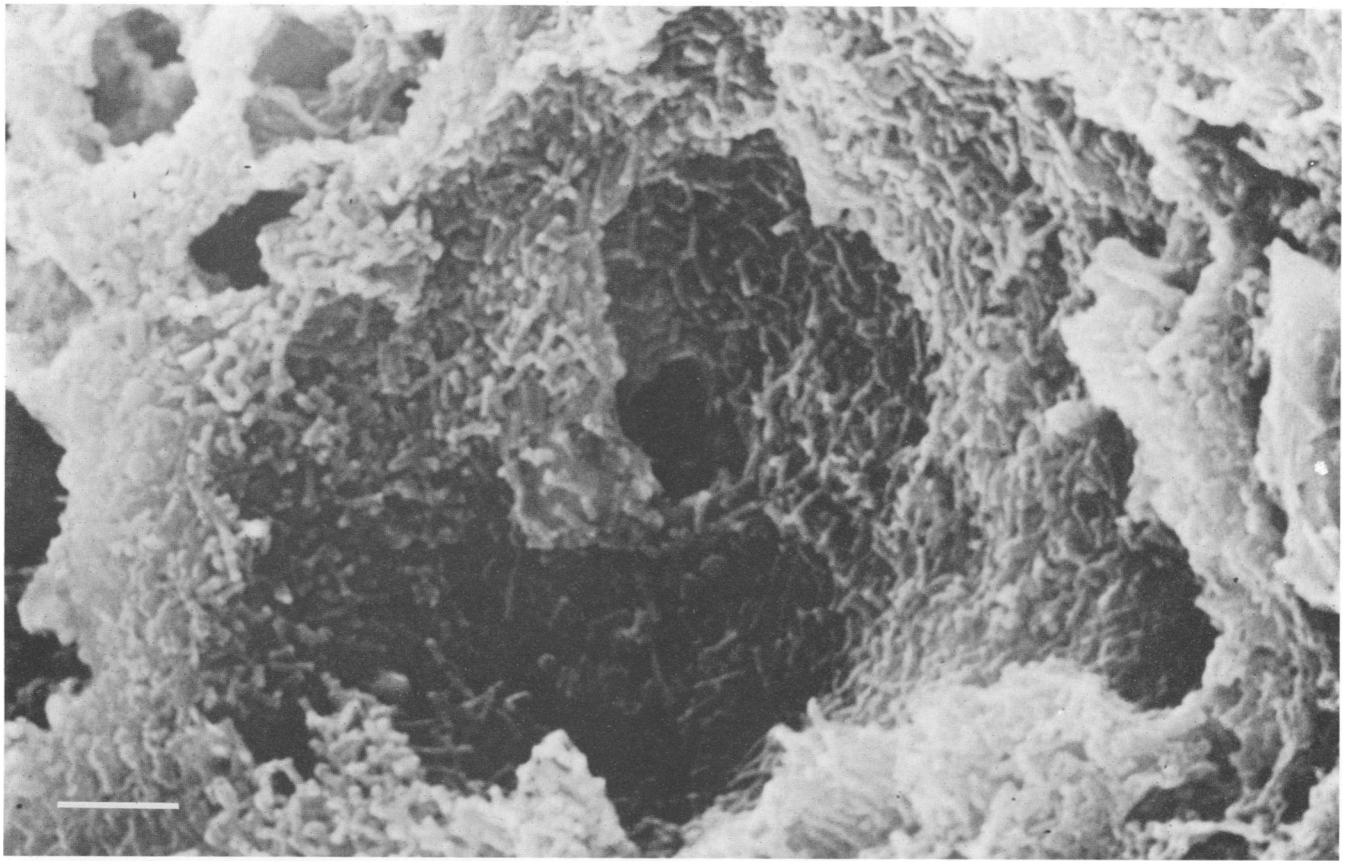

FIG. 9. High magnification of the third layer, revealing that large numbers of bacteria with angular wall structures are found in the walls of the internal cavities. Bar, 5.0 μm.

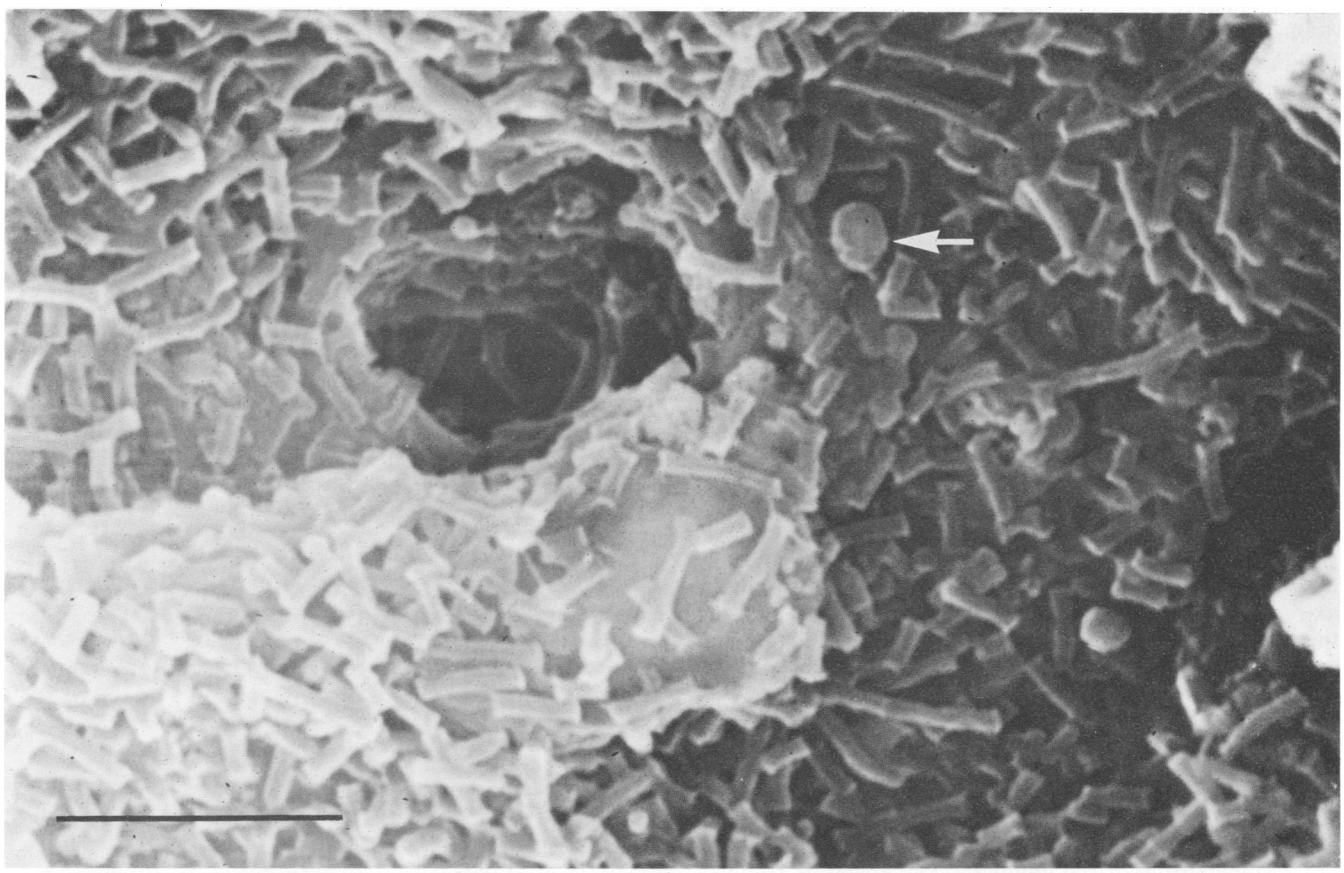

FIG. 10. Organisms in this third layer were surrounded by a condensed extracellular layer. Occasionally, other cell structures (←) were observed in the third layer. Bar, 5.0 μm.

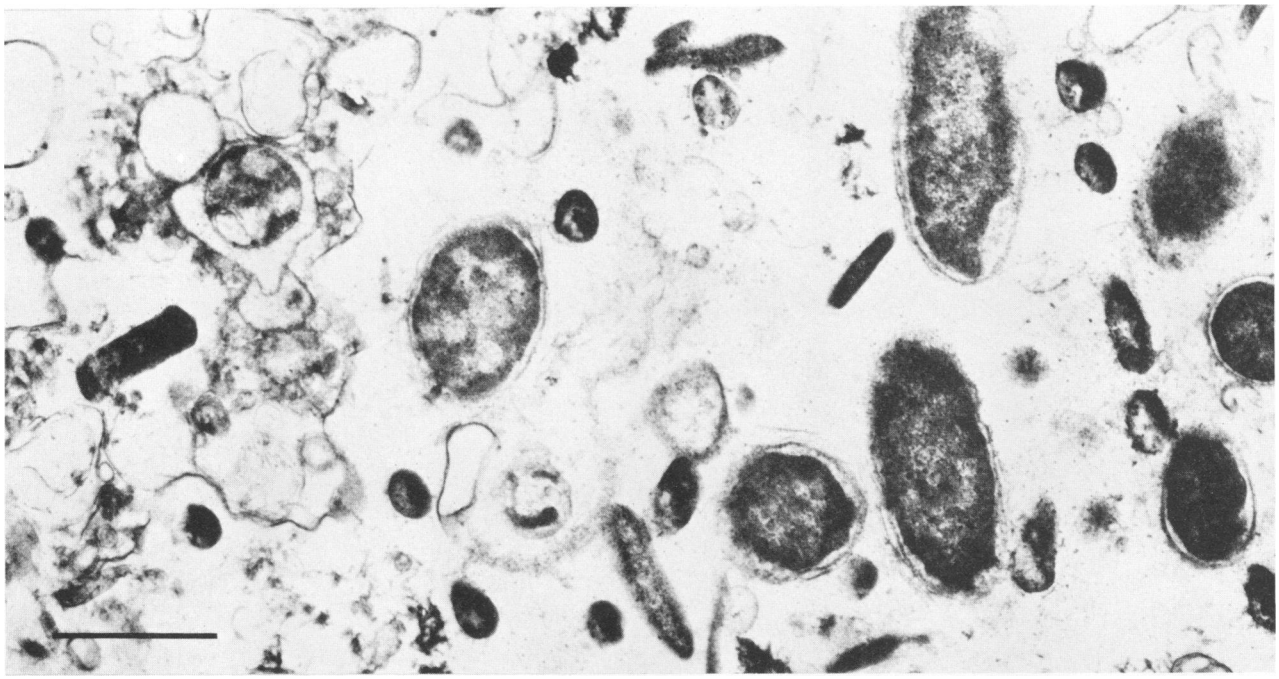

FIG. 11. Transmission electron micrograph showing the diversity of species present at the surface. Bar, 1.0 μm.

Methanothrix soehngenii (16). It was also apparent that a large number of cavities were present in the third layer (Fig. 6). Since the walls of these cavities were surrounded by large numbers of *Methanothrix*-like organisms, it seems plausible that these areas are sites of vigorous gas production. Evidence that the cavities in bacterial granules are sites of gas production has been previously provided (3). Bochem and co-workers (3) have also described three morphologically distinct layers in thermophilic granules maintained with an acetic acid medium. However, each layer consisted of bacteria with only one shape, and this is a major contrast to the diversity of species which we observed in the first two layers of the mesophilic granules. The limited number of species that were present in the fermentor of Bochem and co-workers (3) was probably due to the use of thermophilic conditions and also to the use of a simple compound, acetic acid, as the carbon and energy source.

Extracellular polymer production was observed in each of the three different layers in the granules (Fig. 4, 8, and 10). It has been well documented previously that an extracellular polymer mediates the adhesion of bacteria in natural ecosystems (7). The multiplication of adherent bacteria often results in the formation of microcolonies of morphologically identical cells, because daughter cells are trapped within the extracellular polymer. In a similar manner, one can envision the development of microcolonies in granular sludge. Once cells are adherent to a precursor aggregate, they would divide within the extracellular polymer. If two or more different species were physically associated with each other in a metabolic consortium, the species would continue to propagate in the ordered juxtaposition to form mixed microcolonies. Figure 2 shows a good example of a mixed microcolony present in granular sludge.

Activity measurements (see above) have indicated that organisms capable of the complete conversion of complex substrates to gaseous products are present within the granular sludge. Thus, it is conceivable that the entire granule may act as a single large mixed microcolony in which cross-feeding is occurring between members of a large consortium. However, it is also possible that substrate transfer occurs between individual granules. Similar consor-

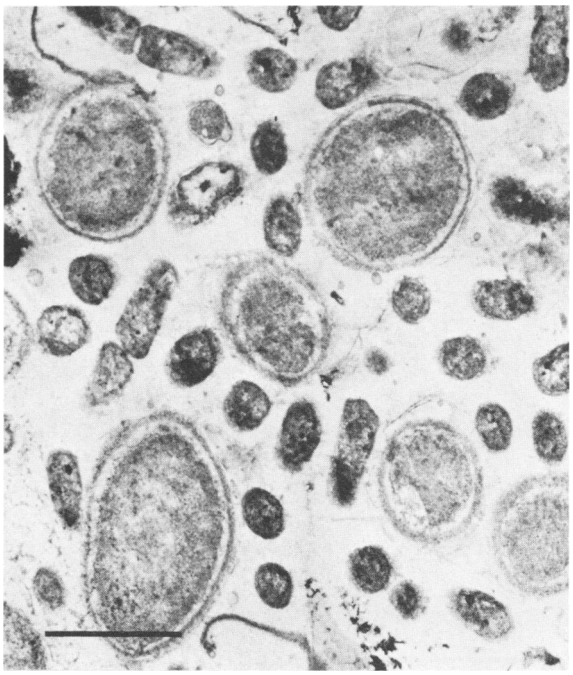

FIG. 12. Thin section obtained at the approximate depth of the second layer showing an arrangement of two types of cell structures in a mixed microcolony. Bar, 1.0 μm.

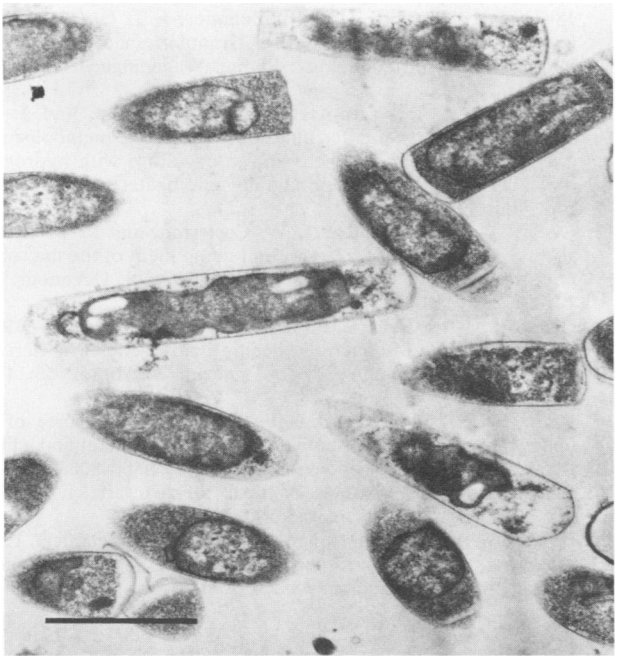

FIG. 13. Large microcolonies of a single rod structure from a depth of 50 to 100 μm. Bar, 1.0 μm.

tia of physiologically related bacteria in metabolic processes including rumen fermentation (29), degradation of chlorinated organic compounds (13), and anaerobic digestion (21) have been previously described.

A number of investigators have described the prominence of *Methanothrix* spp. in anaerobic wastewater systems (9, 27, 28). The presence of *Methanothrix*-like cells in the central core of the granules (Fig. 10) suggests that *Methanothrix* aggregates may function as nucleation centers that initiate granule development (Fig. 15). It is conceivable that a loose mat of *Methanothrix* filaments such as those which develop in pure culture (25) provides an excellent framework that could be colonized by a succession of other organisms. Some of the first colonizing organisms would be those which produce acetate. These bacteria would provide the *Methanothrix* spp. with the required substrate. The acetate producers could include H_2-producing acetogens. This group plays a central role in the anaerobic digestion of organic matter (22). Volatile fatty acids produced by fermentative bacteria are degraded to acetate by the H_2-producing acetogens. However, it is well documented that high concentrations of H_2 inhibit the degradation of propionate and butyrate by this metabolic group (4, 5, 23). Thus, this metabolic group requires a syntrophic association with H_2-using bacteria in order to use substrates such as propionate and butyrate. The presence in granular sludge of organisms involved in such syntrophic associations is confirmed by the degradation of propionate in our granular sludge samples. Further support of this hypothesis is found in a complementary study performed in our laboratory by A. Pauss et al. (Pauss et al., Appl. Microbiol. Biotechnol., in press). On the basis of Gibbs free energy changes, it was concluded that only microbial microniches within the granular structure would be environments in which the hydrogen concentration was low enough to permit the degradation of propionate. Thus, one can envision the formation of a second layer around the *Methanothrix* mat that would include H_2-producing acetogens and H_2-consuming organisms. The adhesion of fermentative bacteria to the miniaggregate to form the exterior layer of the granule would provide contact between this metabolic group and its substrates, which are present in the external milieu. The activity measurements confirm the presence of this metabolic group in the granule structure. The products of this metabolic group would then serve as substrates to the underlying acetogens. In addition, the presence of methanogenlike organisms (Fig. 3) in the exterior layer suggests that H_2-using organisms could consume any free hydrogen before it penetrated into the second layer. Hydrogen-using organisms present in the second layer would then be able to remove any remaining hydrogen produced by the acetogens so that a high level of metabolic activity by the acetogens would occur. The removal of an analogous gas in biofilms has been described elsewhere (1, 6). It has been documented that aerobic and facultative anaerobic bacteria present in the exterior layer of a biofilm will create an O_2 gradient such that strict anaerobes can flourish in the deeper layers of the biofilm. Such a three-layered aggregate would be a very complete and stable metabolic arrangement that would create optimal environmental conditions for all its members. The resulting high levels of metabolic activity and cell growth should permit the aggregate to reach the size of sludge granules.

We are suggesting one developmental hypothesis which appears to most completely fit the presented data. Another hypothesis would be that granules develop as miniaggregates

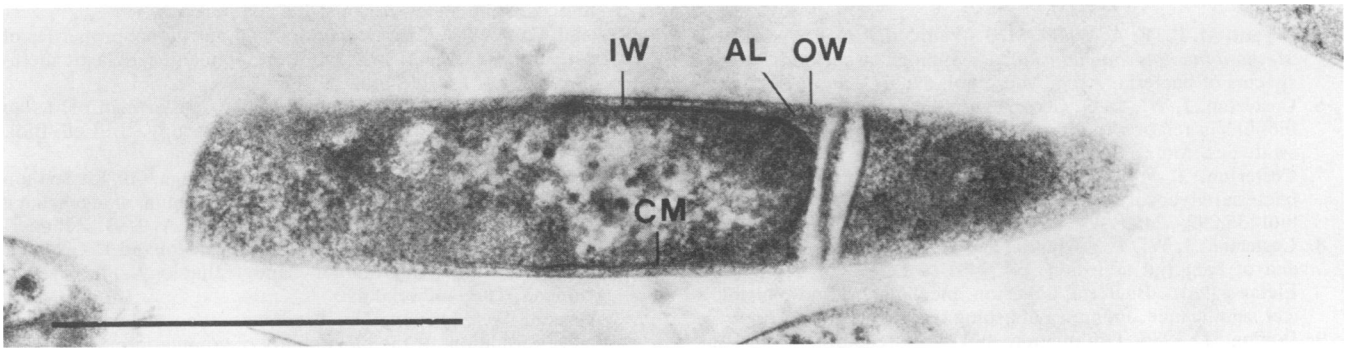

FIG. 14. Detailed examination of this morphotype reveals that the walls are multilayered. CM, Cell membrane; IW, inner wall; AL, amorphous layer; OW, outer wall. Bar, 1.0 μm.

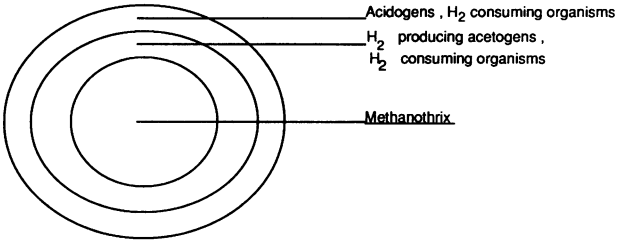

FIG. 15. Proposed granule, showing the partitioning of metabolic groups into separate granular layers. See text for detailed description.

which contain different microbial populations and adhere to one another. However, our results, particularly the observation of a large central core that consists primarily of a single species, do not fit the latter hypothesis. In this study we have examined the ultrastructure of sludge granules that were present in an upflow anaerobic sludge blanket reactor at one particular time. Future studies will endeavor to induce granulation by using the tentatively identified microorganisms. Thus, we will be able to monitor species interactions, biochemical activity, and aggregate ultrastructure at various stages of development.

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LITERATURE CITED

1. **Alleman, J. E., J. A. Veil, and J. T. Canaday.** 1982. Scanning electron microscope evaluation of rotating biological contactor biofilm. Water Res. **16**:543–550.
2. **Arcand, Y., M. DesRochers, C. Chavarie, and S. R. Guiot.** 1989. Effect of the hydraulic regime on the granule size distribution in an upflow anaerobic reactor, p. 357–370. *In* C. A. Cole and D. A. Long (ed.), Hazardous and industrial wastes. Proceedings of the 21st Mid-Atlantic Industrial Waste Conference. Technomic Publishing Co., Lancaster, Pa.
3. **Bochem, H. P., S. M. Schoberth, B. Sprey, and P. Wengler.** 1982. Thermophilic biomethanation of acetic acid: morphology and ultrastructure of a granular consortium. Can. J. Microbiol. **28**:500–510.
4. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. **40**:626–632.
5. **Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe.** 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Microbiol. **59**:20–31.
6. **Costerton, J. W., G. G. Geesey, and P. A. Jones.** 1988. Bacterial biofilms in relation to internal corrosion monitoring and biocide strategies. Materials Performance **27**:49–53.
7. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35**:299–324.
8. **Costerton, J. W., T. J. Marrie, and K.-J. Cheng.** 1985. Phenomena of bacterial adhesion, p. 3–43. *In* D. C. Savage and M. Fletcher (ed.), Bacterial adhesion, mechanisms and physiological significance. Plenum Publishing Corp., New York.
9. **Dolfing, J.** 1986. Granulation in UASB reactors. Water Sci. Technol. **18**:15–25.
10. **Dubourguier, H. C., G. Prensier, and G. Albagnac.** 1988. Structure and microbial activities of granular anaerobic sludge, p. 18–33. *In* G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Puduc, Wageningen, The Netherlands.
11. **Dwyer, D. F., E. Weeg-Aerssens, D. R. Shelton, and J. M. Tiedje.** 1988. Bioenergetic conditions of butyrate metabolism by a syntrophic anaerobic bacterium in coculture with hydrogen-oxidizing methanogenic and sulfidogenic bacteria. Appl. Environ. Microbiol. **54**:1354–1359.
12. **Geesey, G. G., R. Mutch, J. W. Costerton, and R. B. Green.** 1978. Sessile bacteria: an important component of the microbial population in small mountain streams. Limnol. Oceanogr. **23**: 1214–1223.
13. **Genthner, B. R., W. A. Price II, and P. H. Pritchard.** 1989. Characterization of anaerobic dechlorinating consortia derived from aquatic sediments. Appl. Environ. Microbiol. **55**:1472–1476.
14. **Guiot, S. R., and L. van den Berg.** 1985. Performance of an upflow anaerobic reactor combining a sludge blanket and a filter treating sugar waste. Biotechnol. Bioeng. **27**:800–806.
15. **Henrici, A. T.** 1933. Studies of freshwater bacteria. I. A direct microscopic technique. J. Bacteriol. **25**:277–286.
16. **Huser, B. A., K. Wuhrmann, and A. J. B. Zehnder.** 1982. *Methanothrix soehngenii* gen. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **132**: 1–9.
17. **Jones, W. J., J.-P. Guyot, and R. S. Wolfe.** 1984. Methanogenesis from sucrose by defined immobilized consortia. Appl. Environ. Microbiol. **47**:1–6.
18. **Kjelleberg, S.** 1984. Adhesion to inanimate surfaces, p. 71–84. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag KG, Berlin.
19. **Lettinga, G., P. Hulshoff, W. Wiegant, W. de Zeeuw, S. W. Hobma, P. Grin, R. Roersma, S. Sayed, and A. F. M. van Velsen.** 1983. Upflow sludge blanket processes, p. 139–158. *In* R. L. Wentworth (ed.), Proceedings of the Third International Symposium on Anaerobic Digestion. Evans and Faulkner, Inc., Watertown, Mass.
20. **Lettinga, G., A. F. M. van Velsen, W. Hobma, W. J. de Zeeuw, and A. Klapwijk.** 1980. Use of the upflow sludge blanket (USB) reactor concept for biological waste water treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22**:699–734.
21. **Mah, R. A., D. M. Ward, L. Baresi, and T. L. Glass.** 1977. Biogenesis of methane. Annu. Rev. Microbiol. **31**:309–342.
22. **McInerney, M. J.** 1986. Transient and persistent associations among prokaryotes, p. 293–338. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature, vol. 2. Plenum Publishing Corp., New York.
23. **McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton.** 1981. *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. **41**:1029–1039.
24. **Paerl, H. W.** 1980. Attachment of microorganisms to living and detrital surfaces in freshwater systems, p. 375–402. *In* G. Bitton and K. C. Marshall (ed.), Adsorption of microorganisms to surfaces. John Wiley & Sons, Inc., New York.
25. **Patel, G. B.** 1984. Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic, aceticlastic methanogen. Can. J. Microbiol. **30**:1383–1396.
26. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
27. **Rinzema, A., J. van Lier, and G. Lettinga.** 1988. Sodium inhibition of acetoclastic methanogens in granular sludge from a USAB reactor, p. 216–222. *In* G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, The Netherlands.
28. **Wiegant, W. M.** 1988. The "spaghetti theory" on anaerobic sludge formation, or the inevitability of granulation, p. 146–152. *In* G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and

technology. Pudoc, Wageningen, The Netherlands.
29. **Wolin, M. J.** 1979. The rumen fermentation: a model for microbial interactions in anaerobic ecosystems, p. 49–78. *In* M. Alexander (ed.), Advances in microbial ecology, vol. 3. Plenum Publishing Corp., New York.
30. **Zehnder, A. J. B., K. Ingvorsen, and T. Marti.** 1982. Microbiology of methane bacteria. *In* D. E. Hughes, D. A. Stafford, B. I. Wheatley, W. Baader, G. Lettinga, E. J. Nyns, W. Verstraete, and R. L. Wentworth (ed.), Anaerobic digestion 1981. Elsevier Biomedical Press, Amsterdam.
31. **Zeikus, J. G., and V. G. Bowen.** 1975. Comparative ultrastructure of methanogenic bacteria. Can. J. Microbiol. **21**:121–129.